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Metabolic inhibitors, elicitors, and precursors as tools for probing yield limitation in taxane production by Taxus chinensis cell cultures
AU Srinivasan, V.; Ciddi, V.; ***Bringi, V.*** ; Shuler, M. L.
CS School of Chemical Engineering, Cornell University, Ithaca, NY, 14853, USA
SO Biotechnol. Prog. (1996), 12(4), 457-465

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Large scale production of secondary metabolites using plant cell cultures: Opportunities, realities and challenges.
AU Venkat, K.; ***Bringi, V.*** ; Kadkade, P.; Prince, C.
CS Phyton Inc., Itacha, NY 14850 USA
SO Abstracts of Papers American Chemical Society, (1997) Vol. 213, No. 1-3, pp. AGFD 54.
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SO Book of Abstracts, 213th ACS National Meeting, San Francisco, April 13-17 (1997), AGFD-054 Publisher: American Chemical Society, Washington, D. C.

Production of ***taxol*** by cell culture of Taxus. For development of techniques for industrial production
AU Hara, Yasuhiro; Yukimune, Yukihito
CS Mitsui Petrochem. Ind., Ltd., Yamaguchi, 740, Japan
SO Farumashia (1996), 32(7), 806-809
CODEN: FARUAW; ISSN: 0014-8601
DT Journal; General Review
LA Japanese

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TI Effect of picloram and methyl ***jasmonate*** on growth and ***taxane*** accumulation in callus culture of Taxus X media var. Hatfieldii.
AU Furmanowa, M.; Glowniak, K.; Syklowska-Baranek, K.
SO Plant cell, tissue and organ culture, 1997. Vol. 49, No. 1. p. 75-79
Publisher: Dordrecht, The Netherlands : Kluwer Academic Publishers.

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TI Large-scale plant cell culture
AU Roberts, Susan C.; Shuler, Michael L.
CS Sch. Chemical Eng., Cornell Univ., Ithaca, NY, 14853-5201, USA
SO Curr. Opin. Biotechnol. (1997), 8(2), 154-159

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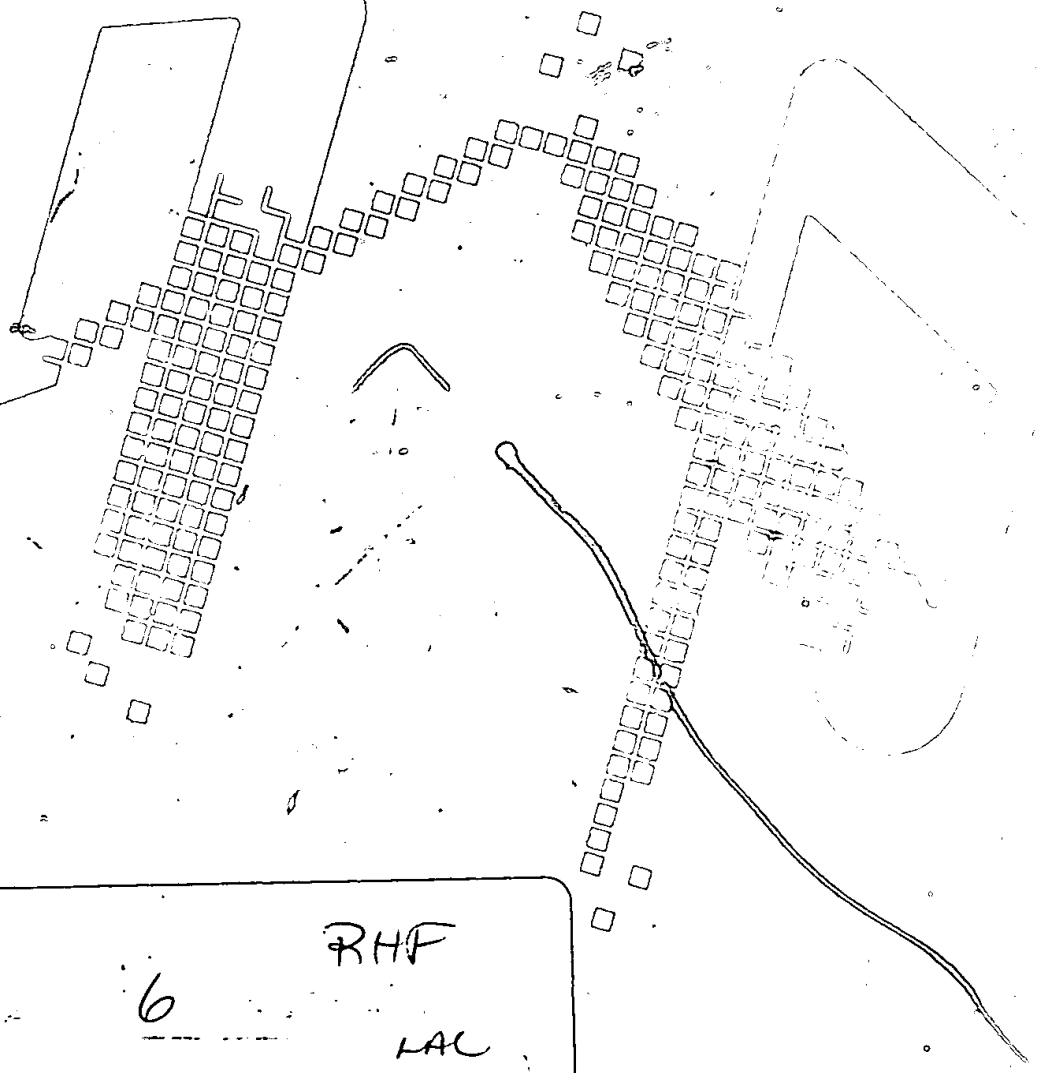

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Metabolic Inhibitors, Elicitors, and Precursors as Tools for Probing Yield Limitation in Taxane Production by *Taxus chinensis* Cell Cultures

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Inhibition of biosynthetic enzymes and translation and translocation processes, elicitation, and precursor feeding were used to probe biosynthetic pathway compartmentation, substrate–product relationships, and yield limitation of the diterpenoid taxanes in cell cultures of *Taxus chinensis* (PRO1-95). The results suggest the following: (i) the source of isopentenyl pyrophosphate in taxane production is likely plastidic rather than cytoplasmic; (ii) baccatin III may not be a direct precusor of Taxol (Taxol is a registered trademark of Bristol-Myers Squibb for paclitaxel); (iii) baccatin III appears to have cytoplasmic and plastidic biosynthetic components, while Taxol production is essentially plastidic; and (iv) arachidonic acid specifically stimulates Taxol production but does not have a significant effect on baccatin III yield. Semiempirical mathematical models were used to describe these results and predict potential yield-limiting steps. Model simulations suggest that, under current operating conditions, Taxol production in *Taxus chinensis* (PRO1-95) cultures is limited by the ability of the cells to convert phenylalanine to phenylisoserine rather than by the branch-point acyl transferase. This result is supported by the lack of improvement of Taxol yield by feeding phenylalanine or benzoylglycine. The methods described in this article, while specifically expanding our knowledge of taxane production in PRO1-95 cultures, could be generally useful in investigating complex aspects of secondary metabolic pathways in plant cell cultures, especially when details of the pathway and compartmentation are sparse.

Introduction

Metabolic pathway engineering of plant cells for improving the yields of valuable secondary compounds generally has not been possible. Yet, the intelligent manipulation of plant cell cultures in the laboratory entirely depends on our ability to identify key points of metabolic flux control. Often this task has to be undertaken without the resource of adequate information regarding biochemistry, regulation, genetics, and compartmentation. With the therapeutic and commercial success of Taxol, there is renewed interest in plant secondary metabolites, and as novel compounds continue to be discovered, there will be an even greater interest in the use of plant cell cultures for their production.

Obtaining detailed information about the biosynthetic pathway along with the relevant information about regulation and compartmentation can take decades of patient work. Typically, the immediate need to produce large quantities of valuable drugs such as Taxol cannot be reconciled with this time frame. Therefore, it is vital to be able to use established tools to quickly construct a working model of the pathway and regulation and then integrate this knowledge into an iterative strategy for pathway manipulation and yield improvement.

Taxol and related taxanes are derivatized diterpenes derived from the universal diterpene precursor geranylgeranyl pyrophosphate (GGPP). Figure 1 shows a biogenetic scheme constructed from the results of Croteau *et al.* (1995) and Fleming *et al.* (1994). It has been proposed by Koepp *et al.* (1995) that GGPP is cyclized to an olefin (taxadiene) intermediate, and several oxygenation and acylation reactions follow to complete the structure of Taxol. In support of this hypothesis, Croteau *et al.* (1995) have identified a diterpene (taxadiene) cyclase and a putative NADPH and O_2 dependent, microsomal cytochrome P450 oxygenase believed to catalyze the early steps of Taxol biosynthesis from GGPP. Stoebel *et al.* (1992) first demonstrated that label from [^{14}C]phenylalanine was incorporated into the phenylisoserine side chain at C-13. Subsequently, Fleming *et al.* (1994) proposed that phenylisoserine is synthesized from phenylalanine via β-phenylalanine. Further, they suggest that the benzoyl moiety is finally attached to debenzoyltaxol, as shown in Figure 1. Fleming *et al.* (1994) have also shown that tritium label from [^{3}H]-baccatin III (Figure 1) is incorporated into Taxol by using plant segments. The results of Stroebel *et al.* (1992), showing that radiolabeled carbon from glucose, acetate, leucine, and phenylalanine is incorporated into Taxol by *Taxus brevifolia* bark, and the results of Zamir *et al.* (1992), showing labeling of Taxol by [^{14}C]mevalonic acid in cell-free homogenates of *Taxus canadensis* tissue, are generally consistent with conventional mevalonate-derived isoprenoid biosynthetic pathways and the biogenetic scheme in Figure 1. Although we believe that this biogenetic scheme (Figure 1) is the most likely one, recent developments with studies of isoprenoid synthesis in

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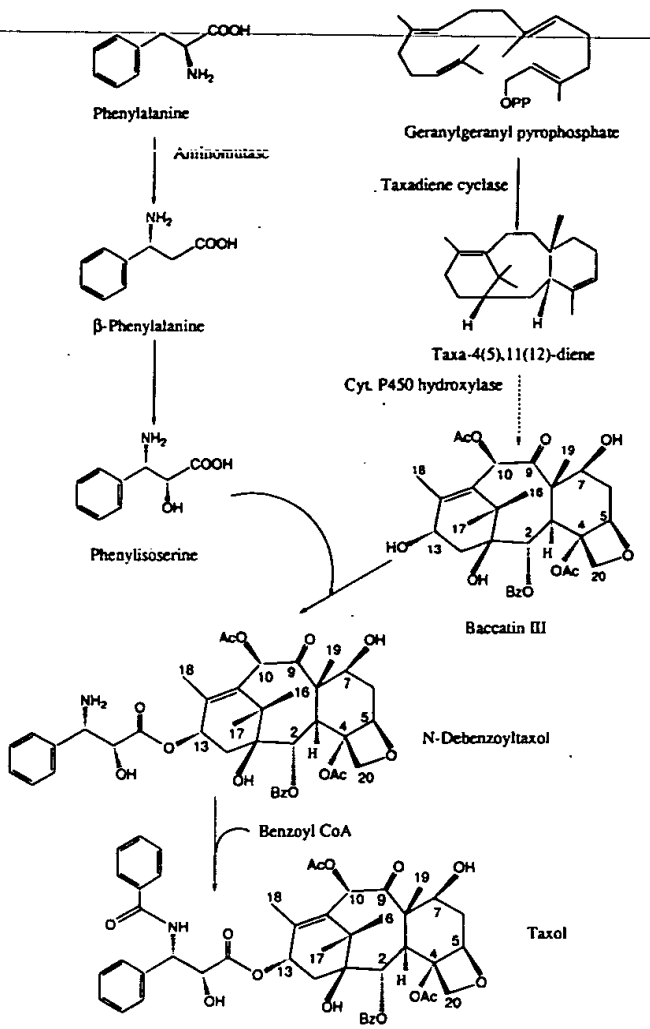

Figure 1. Biogenetic scheme for baccatin III and Taxol constructed from the works of Croteau *et al.* (1995) and Fleming *et al.* (1994). Dashed lines indicate multiple reaction steps.

bacteria (Rohmer *et al.*, 1993) demonstrate that unconventional pathways for isoprenoid synthesis exist.

The source of isopentenyl pyrophosphate (IPP) for the synthesis of GGPP in the plastid is uncertain. Although some (e.g., Kreuz and Kleinig, 1984) suggest that cytoplasmically derived IPP is the sole source of IPP in the plastids, the absence of concrete evidence for an IPP translocator coupled with the experimental difficulties of obtaining unperturbed, purified plastids raises doubts about this assertion. Moreover, the temporal and developmental regulation of isoprenoid pathways (Schultz and Schultze-Siebert, 1989) makes precise assignments of localization and activity difficult. It is generally accepted that GGPP biosynthesis occurs in the plastids of plant tissues (Kleinig, 1989). It is also known that glycolysis and early isoprenoid biosynthesis (up to farnesyl pyrophosphate) occur in other cellular compartments in addition to the plastids.

On the basis of this information, taxane biosynthesis can reasonably be expected to involve the transport of primary carbon/nitrogen precursors into the plastids where at least part of the biosynthesis would take place. On the basis of the involvement of cytochrome P450 oxygenases (Croteau *et al.*, 1995), it may be expected that hydrocarbon or partially oxygenated intermediates may be transported from the plastids to other organelles, for example, the endoplasmic reticulum, resulting in the formation of a baccatin III (Figure 1) type molecule. However, plastids are equipped with enzymatic machinery to introduce oxygen functions into isoprenoids (Kleinig, 1989), so it is not essential that transport to the ER must occur. Aromatic amino acid biosynthesis is largely plastidic, but there is believed to be a cytoplasmic pathway as well (Morris *et al.*, 1989), so that the synthesis and/or addition of the side chain could occur in either the plastids or the endoplasmic reticulum. Finally, the completed Taxol/taxane molecule could be transported to cellular storage sites such as the vacuoles or excreted from the cells. Metabolite transport between cellular compartments and intraorganellar biochemistry could be key components of taxane production. This concept is exemplified in monoterpene indole alkaloid biosynthesis in *Catharanthus roseus* (Meijer *et al.*, 1993).

In contrast to uncompartmented bacterial systems, this aspect of compartmentation and translocation in plant cells greatly complicates rational metabolic manipulation. To simplify this complex scenario in *Taxus* species cell cultures, we addressed ourselves to the following specific questions: What is the source of IPP in Taxol biosynthesis? Are Taxol and other taxanes made in the same cellular compartment? Must baccatin III be a direct biosynthetic precursor of Taxol? Is the synthesis/attachment of the side chain rate limiting, or is the building of the primary four-ring taxane skeleton rate limiting? Are there simple strategies that can be used to manipulate product levels?

In this paper, we demonstrate an approach to this strategy of semirational manipulation of metabolic pathways in plant cell cultures. We have used inhibitors of carrier proteins involved in metabolite translocation, translation inhibitors such as cycloheximide and streptomycin, and inhibition of oxygenase enzymes to construct conceptual schemes of carbon flow and cellular compartmentation of the biosynthetic pathway. By using simple semiempirical mathematical models to describe these conceptual schemes, we have predicted potential rate-limiting steps. These predictions are consistent with data from elicitation and precursor feeding experiments.

Materials and Methods

Cell Line and Maintenance. PRO1-95 (*Taxus chinensis*) cell suspensions were kindly provided by Phyton, Inc., for the duration of these studies. A closely related culture and its manipulation for Taxol production has been described (Bringi *et al.*, 1995). The cell cultures were maintained in Gamborg's B5 medium containing 1% sucrose, 5 μM naphthylacetic acid, and 0.01 μM benzyladenine. pH was adjusted to 5.6 before autoclaving. Sterile ascorbic acid (100 mg/L) and glutamine (2 mM) were added to the medium after sterilization. A transfer period of 1 week using 5% (w/v) inoculum was employed. Cultures were maintained in the dark at 25 °C.

Culture Conditions. Multiwell, polystyrene plates (Srinivasan *et al.*, 1996) were used to facilitate testing many culture conditions simultaneously. One-week-old cell suspensions were filtered through Miracloth, and by using an inoculum of 20% (w/v), the cells were transferred into an induction medium consisting of one-fourth strength B5 medium containing 5% sucrose and 10 μM 2,4-D (2,4-dichlorophenoxyacetic acid) and 2.5 μM zeatin. One gram fresh weight of cells was placed in each well of a six-well plate, and 4 mL of induction medium was added. The plates were lightly sealed with Parafilm and incubated in a shaking incubator (120 rpm) in the dark at 25 °C.

Culture Manipulations. All chemicals were the highest grade available from Sigma. ABT (1-aminobenzotriazole) and arachidonic acid were dissolved in etha-

Table 1. Effect of Cycloheximide and Streptomycin on Taxane Production[a]

conditions	baccatin III (μM)	taxol (μM)
control	3.01 ± 0.93	4.80 ± 0.84
cycloheximide		
1 μM	4.05 ± 0.11	4.70 ± 0.41
10 μM	ND[b]	4.19 ± 0.74
streptomycin		
1 μM	ND[b]	3.45 ± 0.61
10 μM	ND[b]	ND[b]

[a] Inhibitors were added at inoculation, and cultures were harvested on day 15. [b] Level too low for spectral confirmation and therefore not detectable.

nol. All other chemicals were dissolved in deionized water with or without the addition of a few drops of acid or base. The pH of all solutions was adjusted to approximately 5.6 before administering.

Cell Growth and Product Measurement. Fifteen days after inoculation into the induction medium, the wells were harvested. Medium was carefully pipetted out, and the fresh weight of the remaining cells was measured by scooping the remaining cell mass into a tared glass jar. Whole broth from each well was freeze–thawed to disrupt the cell walls and membranes and then extracted in three volumes of acidified methanol (0.1% acetic acid) by sonication and standing overnight. Taxane content in the cell-free supernatant was measured by Phyton, Inc., using reversed-phase HPLC and diode array detection. Modifications of the methods of Witherup *et al.* (1989) and Ketchum and Gibson (1993) were employed. A Phenomenex Curosil G column (4.6 × 250 mm, 6 μm) with a Curosil G guard column (4.6 × 30 mm, 6 μm) was used. The gradient profile began at 10 mM KH_2PO_4 taken to pH 3.5 with trifluoroacetic acid/acetonitrile (60:40). The gradient progressed linearly to a 25:75 mixture over 12 min and was held at 25:75 for 5 min to wash the column. Twenty-five microliters of sample was injected. Retention times of baccatin III and Taxol were 5 and 8 min, respectively. The identities of individual taxanes were confirmed by comparing UV spectra with those of authentic standards (kindly provided by the National Cancer Institute). The identities of taxanes produced from cell cultures were also confirmed independently by tandem mass spectroscopy by Phyton, Inc.

Data Analysis and Statistics. All data represent the mean and standard deviation of six points (from six individual wells). The data were analyzed by one-way ANOVA (analysis of variance), and the differences between means were tested by Tukey's HSD test. All reported differences are significant at least at $P = 0.05$.

Results and Discussion

Localization of Baccatin III and Taxol Biosynthesis and Source of IPP. Since isoprenoid biosynthesis occurs in more than one cellular compartment, it is important to understand the localization of taxane production before addressing yield improvement strategies such as precursor feeding or elicitation. Cycloheximide (CH) and streptomycin (SS) have been used widely as tools to distinguish between cytoplasmic and plastidic mRNA translation events (Bhadula and Shargool, 1991; Mita *et al.*, 1995). Therefore, if taxane production had a significant plastid component, we would expect the process to be sensitive to SS, and if the biosynthesis was predominantly cytoplasmic, we would expect the process to be sensitive to CH. Table 1 shows the effect of CH and SS on taxane production. At 1 μM CH, no significant change in baccatin III yield was observed, while at 10 μM, baccatin III production was completely inhibited.

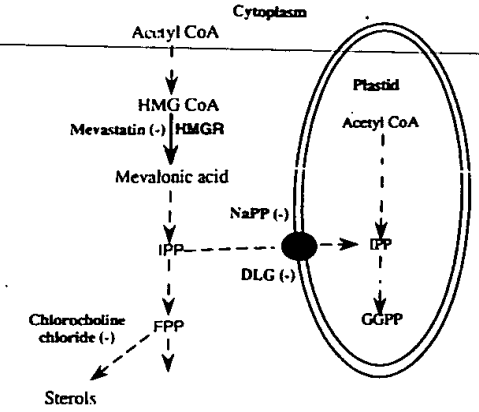

Figure 2. Source of isopentenyl pyrophosphate in plastid isoprenoid biosynthesis. Translocators on the plastid envelope membrane (shaded circle) have been suggested as the import mecahnism. Dashed lines indicate more than one reaction step.

Taxol production was unaffected by CH. However, Taxol production, which was barely affected at the lower SS concentration, was completely inhibited at the higher SS concentration. SS at 1 and 10 μM also completely inhibited baccatin III production. These results suggest that baccatin III production has both cytoplasmic and plastidic components, while Taxol production is localized mainly in the plastids.

The autonomy of plastids for the biosynthesis of mevalonic acid (Moore and Shephard, 1978; Camara *et al.*, 1983; Bach, 1987), IPP (Reddy and Das, 1987), and higher isoprenoids (Camara and Moneger, 1982; Schulze-Siebert and Schultz, 1987) has been amply demonstrated. However, Camara and Moneger (1982), Kreuz and Kleinig (1984), Soler *et al.* (1993), and others have shown that plastids are capable of IPP uptake. On the basis of their observation that IPP accumulates in plastids against a concentration gradient, Soler *et al.* (1993) have suggested the involvement of a specific translocator. If IPP for taxane production is translocated via the phosphate or adenylate (ADP/ATP) translocators (Flügge and Heldt, 1991; Edwards and Walker, 1983) on the plastid envelope (Figure 2 illustrates this process diagrammatically), then its uptake, and therefore taxane production, would be sensitive to competitive inhibition of the translocators by known inhibitors. To address this possibility, we tested the effect of inhibiting the adenylate and phosphate translocators on taxane production by using the substrate analogs sodium pyrophosphate (NaPP) and D,L-glyceraldehyde (DLG) (Edwards and Walker, 1983). Soler *et al.* (1993) have reported that 2 mM inorganic pyrophosphate lowered [^{14}C]IPP import into *Vitis vinifera* plastids by 67%. At 100 μM or 1 mM, neither of these inhibitors had a significant effect on taxane production. At higher concentrations (e.g., 5 mM), the treatments were toxic and the cultures turned brown. These results suggest that the source of IPP for taxane biosynthesis is largely intrinsic to the plastids.

To further test the hypothesis of a plastidic origin of IPP, we used inhibitors of cytoplasmic isoprenoid biosynthesis. Mevastatin (MVS) (R′ = H) (Mercer, 1993) is a specific inhibitor of 3-hydroxy-3-methylglutarylcoenzyme A reductase (HMGR). Bach *et al.* (1987) and Schindler *et al.* (1984) have shown that, in plant tissues, mevinolin (also known as lovastatin), a close structural analog (R′ = CH_3) of mevastatin, specifically inhibits cytoplasmic HMGR, which would prevent the synthesis of cytoplasmic IPP (Figure 2). Narita and Gruissem (1989) found that treating ripening tomato fruits with mevinolin inhibited the increase in size but not the accumulation of carotenoids. An inhibition of sterol

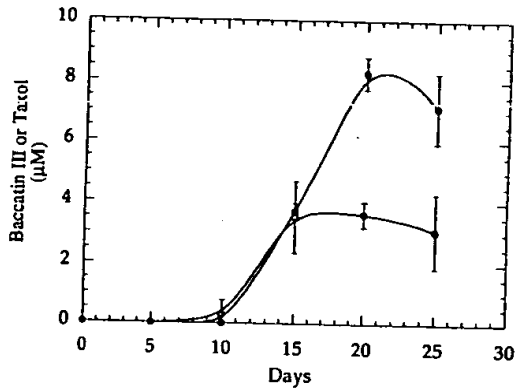

Figure 3. Production kinetics of baccatin III and Taxol in cell suspension cultures of PRO1-95 (*Taxus* sp.): Taxol (●) and baccatin III (○).

biosynthesis (which is cytoplasmic) due to inhibition of HMGR was suggested as the cause of growth inhibition. Since carotenoid biosynthesis is plastidic (Camara *et al.*, 1982), plastidic HMGR activity must also be present. Weissenborn *et al.* (1995) and Yang *et al.* (1991) have shown that solanaeceous plants contain four isoforms of HMGR. Gene sequences for HMGR from several eukaryotic species show extensive active site homology (Weissenborn *et al.*, 1995), suggesting that substrate analogs such as mevinolin or mevastatin would effectively inhibit many isoforms of HMGR provided that the inhibitor has access to the enzyme. Bach (1987) has suggested that mevinolin is incapable of entering the plastids. Therefore, even if *Taxus* cells contain more than one cytoplasmic isoform of HMGR, MVS could be expected to inhibit all cytoplasmic HMGR activity. Chlorocholine chloride (CCC) is also believed to inhibit sterol biosynthesis (Stroebel *et al.*, 1992). We tested the effects of MVS and CCC with and without the addition of mevalonic acid. At 10 or 100 nM MVS (K_I = 1.4 nM) (Mercer, 1993) or 0.1 or 1 mM CCC and 0, 0.1, or 1.0 mM mevalonic acid there was no significant effect on taxane production, indicating that blockage of IPP synthesis in the cytoplasmic compartment was unimportant to taxane synthesis. At 1 μM, MVS was toxic to the cells. The inability of exogenous mevalonic acid to improve Taxol production is also suggestive of compartmentation of Taxol biosynthesis (Floss and Mocek, 1995). A similar lack of effect of exogenous mevalonic acid has been noted in the production of monoterpene indole alkaloids due to localization of key steps of the monoterpene branch in the plastids (Meijer *et al.*, 1993).

Therefore, three separate lines of evidence, the first, from translation inhibitors cycloheximide and streptomycin, the second, from tranlocation inhibitors NaPP and DLG, and the third, from inhibitors of cytoplasmic isoprenoid biosynthesis, collectively suggest that taxane production is predominantly plastidic and that the source of IPP for taxane production is likely plastidic.

Relationship between Baccatin III and Taxol. Cycloheximide (10 μM) was shown to completely inhibit baccatin III production while leaving Taxol production unaffected (Table 1). This suggests a hypothesis where baccatin III need not be a required precursor for the formation of Taxol. This result is condradictory to the results of Fleming *et al.* (1994), who have shown that [^{3}H]-baccatin III is incorporated into Taxol at an absolute incorporation of 0.1% by using plant shoot segments. However, we have two additional lines of information to support a hypothesis where baccatin III need not be a direct precursor of Taxol.

Table 2. Effect of Oxygenase Inhibitors on Taxane Production[a]

condition	baccatin III (μM)	taxol (μM)
control	4.43 ± 0.92	4.49 ± 0.50
ABT		
10 μM	ND[b]	9.08 ± 0.91
100 μM	ND[b]	1.42 ± 0.19
PCA		
10 μM	ND[b]	2.30 ± 0.28
100 μM	ND[b]	ND[b]

[a] Inhibitors were added at inoculation, and cultures were harvested on day 15.ABT, 1-aminobenzotriazole; PCA, 2,5-pyridinedicarboxylic acid. [b] Level too low for spectral confirmation and therefore not detectable.

Figure 3 shows the time course of accumulation of baccatin III and Taxol in PRO1-95 cell suspensions. Baccatin III and Taxol were detectable after day 10. Baccatin III and Taxol accumulated in parallel until day 15, after which baccatin III ceased to accumulate and the level remained constant until day 25. Taxol continued to accumulate until day 20, after which the level remained essentially constant. Maximum concentrations of 3.5 μM baccatin III and about 8 μM Taxol were measured. If baccatin III were a direct precursor of Taxol as suggested by the biogenetic scheme in Figure 1, it could be suggested that side chain addition to baccatin III is rate limiting, and once Taxol production begins, the rate of baccatin III formation decreases until its rate of formation equals its rate of conversion to Taxol. This hypothesis could certainly be tested by straightforward pulse–chase experiments using radiolabeled baccatin III if the material were available to us, but independent synthesis of these two taxanes is consistent with the kinetic observations and does not require the constraining assumption about an exact and sustained balance of baccatin III synthesis and conversion. The hypothesis that baccatin III and Taxol production are at least partially independent is supported by the action of various inhibitors on taxane production (described in the following).

The composition of the taxane molecule, for example, baccatin III, indicates extensive oxidation chemistry following the initial cyclization of GGPP. Croteau *et al.* (1995) have already demonstrated the involvement of a cytochrome (cyt) P450 oxygenase in the oxidation of taxadiene (Figure 1). We tested the response of the taxane spectrum to inhibitors of oxygenase enzymes. Table 2 shows the effects of 1-aminobenzotriazole (ABT), a global suicide inhibitor of cyt P450 monooxygenases (Oritz de Montellano *et al.*, 1981; Reichhart *et al.*, 1982), and 2,5-pyridinedicarboxylic acid (PCA), a competetive inhibitor of α-ketoglutarate dependent dioxygenases (Hanauske-Abel and Guenzler, 1982; Dowell and Hadley, 1992), on taxane production. When measured on day 21 (end point of the experiment), baccatin III levels were undetectably low in response to ABT and PCA treatment. However, at 10 μM ABT, Taxol yield actually increased by 100% over untreated controls. A kinetic experiment was subsequently performed with a higher producing variant of PRO1-95 cells to investigate the temporal action of ABT. The results are shown in Figure 4A,B. We found that the end point data presented in Table 2 were reproduced at this higher level of production, i.e., baccatin III level on day 21 was zero and Taxol levels were roughly twice that of the control. However, in the cultures treated with 10 μM ABT, baccatin III content increased transiently and paralleled the baccatin III content of the control cultures between days 2 and 8 before rapidly decreasing to a low level. In the control culture, the baccatin III level did not change significantly

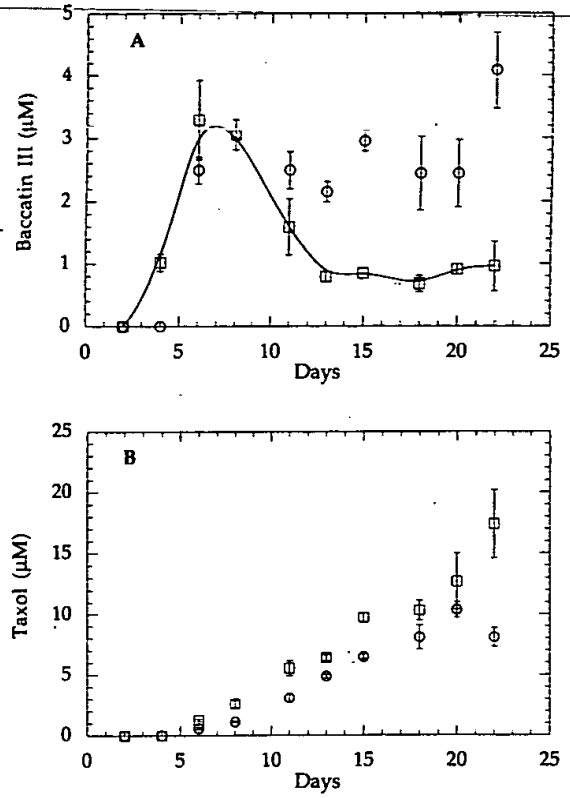

Figure 4. Influence of 1-aminobenzotriazole (ABT) on the kinetics of baccatin III (A) and Taxol (B) accumulation in *Taxus* sp. cell cultures. ABT (10 μM) was added after inoculation of cells into the induction medium.

after day 8. If it is assumed that baccatin III is a direct precursor of Taxol, its rate of synthesis can be calculated from the profiles shown in Figure 4A,B. For example, the net rate of baccatin III accumulation (Figure 4A) equals the rate of synthesis minus the rate of conversion to Taxol (Figure 4B), assuming that baccatin III is not converted to taxanes other than Taxol.

Average rates of synthesis over the production period were calculated. The results show that baccatin III synthesis rates are not very different in control cultures (~0.92 μM day^{-1}) and 10 μM ABT-treated cultures (~0.91 μM day^{-1}). Therefore, it could be suggested that Taxol production in control cultures is limited by the availability of the side chain component and baccatin III is left unconsumed. The use of ABT at the low concentration (10 μM) increases the the rate of conversion of baccatin III to Taxol by causing an increase in the availability of the side chain precursor. Although this hypothesis might appear attractive, a significant aspect of the data is inconsistent with this hypothesis. The substantial increase in Taxol yield is seen toward the end of the culture period, i.e., between days 18 and 22, and does not correspond to the time period of decreasing baccatin III concentration (days 8–12). If indeed the rate of baccatin III conversion is higher in ABT-treated cultures (average rate ~0.88 compared to ~0.55 μM day^{-1} in controls), then we would not expect to see a lag between the decrease in baccatin III levels and the increase in Taxol levels. Therefore, we suggest that the rapid and transient change in baccatin production in response to ABT is unrelated to Taxol production, except possibly in the general context of influencing carbon flux distribution within taxane biosynthesis. At this time, we are not certain of the fate of the rapidly metabolized baccatin III. At 100 μM ABT, Taxol production was inhibited by 42% compared to controls (Table 2). PCA inhibited Taxol production in a dose dependent manner.

The most significant finding is that baccatin III production can be influenced independent of Taxol production. The improvement in Taxol yield at 10 μM ABT, when baccatin III production was suppressed, is consistent with a precursor diversion mechanism from baccatin III production to Taxol production from a common branch point, suggesting that baccatin III is a product of a separate branch of the pathway. Alternatively, ABT could inhibit monooxygenase dependent degradation of Taxol. Our conclusions are also consistent with the lack of improvement in Taxol production in response to baccatin III or 10-deacetylbaccatin III feeding (data not shown), although, in general, yield improvement is not an automatic consequence of precursor feeding experiments due to the possibility of localization of the target reaction in an organelle that is impermeable to the precursor molecule and temporal/developmental regulation of the target enzyme activity.

Therefore, three lines of evidence, namely, the selective inhibition of baccatin III production by cycloheximide without a corresponding effect on Taxol yield, the apparently unrelated responses of baccatin III and Taxol production in response to ABT, and the unchanging kinetic profile of baccatin production with respect to the kinetics of Taxol production, collectively support the hypothesis that baccatin III need not be a direct precursor of Taxol.

Conceptual Models for Carbon Flow in Taxane Production. Figure 5 shows four conceptual models for carbon flow in the production of baccatin III and Taxol. The dotted lines indicate the possibility of more than one step in the respective pathways. The scheme shown in Figure 5A is similar to that suggested in Figure 1, in which baccatin III is a direct precursor of Taxol. Although this model is not consistent with the experimental data reported here, where it was shown that baccatin III production could be influenced without affecting Taxol production, it is included for reference. Figure 5B shows a scheme in which baccatin III is a product of a branch pathway, incapable of participating in taxol biosynthesis. However, since Fleming *et al.* (1994) have shown the incorporation of tritium label from baccatin III into Taxol in the stem tissue of *Taxus brevifolia*, baccatin III is not a dead-end metabolite. Since similar experiments have not been performed with cell cultures, we retain the scenario in Figure 5B as a possible, but unlikely, scheme. Two other more likely explanations may be considered. Either there are stem-specific or species-specific enzyme systems capable of utilizing baccatin III as a precursor, or perhaps baccatin III reenters the Taxol biosynthetic pathway by some as yet undetermined route. The latter possibility is considered in the schemes shown in Figure 5C,D. GGPP is converted to a carbon (C,H,(O)) intermediate X_1, which represents the common branch point metabolite of the Taxol and baccatin III producing branches. In the scheme shown in Figure 5C, X_1 is converted to baccatin III by a reversible reaction. This would allow baccatin III to reenter the pathway via X_1 and therefore accommodate the results of Fleming *et al.* (1994). Also, since baccatin III need not be formed for Taxol production, the results of the previous section are conceptually satisfied. Figure 5D shows a scheme that is consistent with baccatin III being synthesized in a separate branch of the pathway, but having the ability to participate in Taxol biosynthesis by a shunt pathway.

The conceptual models shown in Figure 5B–D were described mathematically by assuming first-order kinetics for each reaction step with respect to individual substrates. On the basis of the equimolar stoichiometry

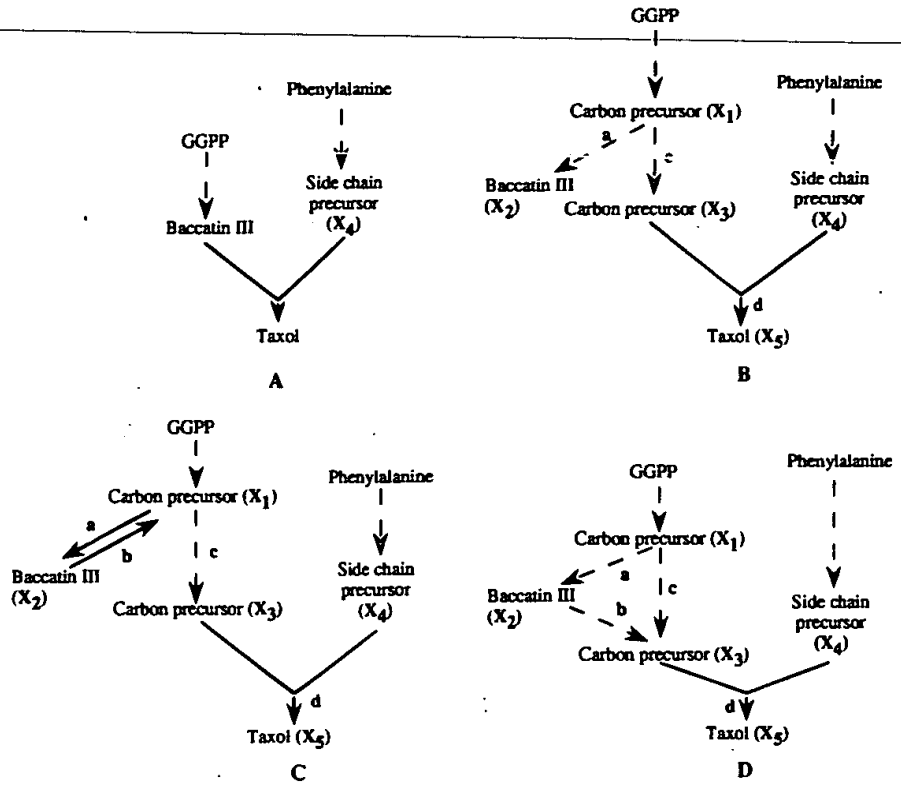

Figure 5. Conceptual models for the flow of carbon in taxane production. GGPP, geranylgeranyl pyrophosphate. Dashed lines indicate the possibility of more than one step in the process.

of the reactions, e.g., 1 mol of GGPP (and therefore X_1) is converted to 1 mol of Taxol or baccatin III and 1 mol of side chain precursor is incorporated into Taxol per mole of carbon intermediate, stoichiometrically sufficient levels of intermediates were assumed as the initial conditions for the carbon precursor (X_1) and the nitrogen precursor (X_4). Semiempirical lumped rate constants (a–d) for the interconversion of metabolites were derived from the observed rates of Taxol and baccatin III production shown in Figure 3. By using 12 and 8 μM as values for X_1 and X_4, respectively, a was estimated as ~0.05 day^{-1}, and d was estimated to be ~0.01 μmol^{-1} day^{-1}. Where necessary, appropriate guesses were made for the unknown rate constants and then minimally adjusted to predict the experimental profiles. For example, an initial estimate for c was made by assuming a pseudo steady state condition for X_3 in Figure 5B after day 15, i.e., the rate of formation of X_3 is equal to its rate of conversion to Taxol, and by assuming values of 12 μM for X_1 and 8 μM X_3 for the calculation. An initial guess for c obtained by this procedure was 0.8 day^{-1}. Since taxane production was not observed before day 10 (Figure 3), i.e., at least some pathway enzymes are not induced ($a = b = c = d = 0$), the model is applicable from day 10 onward. Therefore, the initial conditions for X_1 and X_4 would represent values on day 10 in the overall time course of the culture. Although X_1 and X_4 would change throughout the culture period, the calculation of values for them would involve making additional assumptions for unmonitored upstream reactions. Therefore, the assumption of a complete initial precursor pool is convenient for a preliminary model.

Under conditions of minimal substrate sufficiency, for example, 12 μM X_1 and 8 μM X_4, all three models reasonably predicted the final yields of Taxol and baccatin III, but predicted a much higher rate of accumulation compared to the experimentally observed rates (data not shown). This discrepancy could be explained by considering the product distribution. The models only consider two products, namely, baccatin III and Taxol. In reality, the cultures produce several taxanes, some of which contain side chains and others do not. In these experiments, the total taxane content was typically twice the combined total of Taxol and baccatin III. To satisfy the stoichiometric constraints for a wider spectrum of compounds, it is reasonable to expect that higher substrate concentrations will be necessary. Also, X_1 and X_4 are most likely formed during the synthesis of Taxol and baccatin III, so that initial values may be lower and consequently give rise to lower initial rates. Values of 30 μM for X_1 and 8 μM for X_4 (at $t = 0$) satisfied the rate and yield requirements. Figure 6 compares the model outputs with experimental data. Considering the simplicity of the models, the descriptions of baccatin III and Taxol production, using the values of the parameters listed in Figure 6, are reasonably good. In the following discussion, the models are referred to by their labels in Figure 5. Thus, the model describing baccatin III as an end product will be referred to as model 5B, and so on. The response of the yields of Taxol and baccatin III to augmenting the supply of precursors X_1 and X_4, and increasing the activities of key enzymes (steps a–d) was tested in model simulations. The parameters used in the model simulations are listed in Table 3. The results from these simulations are summarized in the following section.

Prediction of Limiting Processes in Taxol Production. As might be expected, the models predict that the yield of baccatin III is very sensitive to a, X_1, and b. Model 5B suggests that baccatin is a dead-end product. Therefore, X_1 is terminally removed. In models 5C and 5D, baccatin can eventually form X_3. Therefore, in model 5D, baccatin production was found to be very sensitive to b since turnover of baccatin results in the irreversible loss of X_1. However, baccatin III production by model 5C was found to be less sensitive to b since the reaction is reversible around X_1, which in turn can contribute to baccatin formation. As a consequence, in model 5C,

Table 3. Parameters Used in Model (Figure 5) Simulations

model	a (day^{-1})	b (day^{-1})	c (day^{-1})	d ($\mu mol^{-1} day^{-1}$)	range of X_1 ($t=0$) (μM)	range of X_4 ($t=0$) (μM)
5B	0.05, 0.015		0.35, 1.00	0.015, 0.05	30–120	8–120
5C	0.04, 0.2	0.05, 0.15	0.2, 0.8	0.015, 0.05	30–120	8–120
5D	0.04, 0.2	0.05, 0.15	0.2, 0.8	0.015, 0.05	30–120	8–120

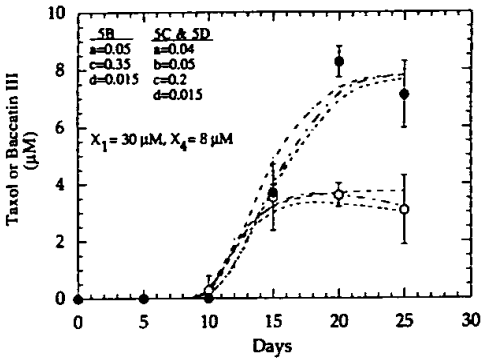

Figure 6. Comparison of model outputs (dashed lines) with experimental data (circles): Taxol (●) and baccatin III (○). Models refer to those shown in Figure 5. Since the model predictions are very similar, individual models are not identified in this figure.

Taxol production was sensitive to increases in b and c especially when a was increased, since increasing either c or b increases the production of X_3. In model 5D, an increase in c was found to offset the depletion of intermediate due to an increase in a, but in model 5C, since baccatin III turnover is reversible, changes in a did not affect Taxol production significantly.

The most significant and nonintuitive prediction from the models was that Taxol yield depends solely on the levels of carbon (X_1) and nitrogen (X_4) intermediates and not on the activity of the side chain transferase. Yield is alternately limited by these substrates depending on their levels, for example, if X_1 is determined to be currently limiting, then increasing the availability of X_1 will result in an improvement in Taxol yield until X_4 becomes the limiting precursor. Further yield improvement will be possible by increasing the availability of X_4. By assuming that our estimates of intermediate concentrations are representative of actual intracellular levels, all three models predict that, under our current operating conditions, the availability of the side chain precursor X_4 limits the yield of Taxol.

Integration of Information and Strategies for Yield Improvement. Metabolic flux depends not only on precursor availability at the site of the reaction but also on enzyme activity. With some knowledge of compartmentation and regulation, substrate supply limitations could be alleviated by feeding suitable precursors at the appropriate time. Similarly, elicitation by biotic or abiotic elicitors could be effectively used to increase the activities of enzymes associated with secondary metabolism (Yoshikawa *et al.*, 1993). Elicitation could be particularly useful in situations where the specific enzymes are not yet known. Therefore, identification of the rate-limiting process, i.e., substrate supply or enzyme activity, is crucial to rational yield improvement.

Since the models predict that, under current operating conditions, the availability of side chain precursor X_4 is rate limiting in Taxol production, we attempted to remedy this limitation by feeding phenylalanine and a potential precursor benzoylglycine (Fett-Neto *et al.*, 1994). The amino acids (0.2 mM each) were added on day 1, and the cultures were harvested on day 15. A 50% increase in Taxol yield was measured. However, this yield could not be improved by feeding higher concentrations (1–5 mM) of precursors. To address the possibility of temporal control of the relevant pathway enzymes, we also tried amino acid addition on day 7. No improvement over controls was measured. This result suggested that the activity of an enzyme involved in X_4 biosynthesis could be rate limiting or that the exogenous precursors could not enter the appropriate biosynthetic pathway. We tried elicitation to address this problem. From several potential elicitors, we identified arachidonic acid as an effective elicitor of Taxol production. Arachidonic acid has been shown to stimulate isoprenoid production in potato (Bostock *et al.*, 1981; Choi and Bostock, 1994). At 5 $\mu g/g$, arachidonic acid increased Taxol production dramatically (9.3-fold) (Table 4), but did not (statistically insignificant) improve baccatin III yield. The control cultures produced low levels of Taxol in these experiments. Although it could be pointed out that arachidonic acid was effective at a low basal level of Taxol production, we have repeatedly observed stimulations of 2–3-fold of Taxol yield in cell cultures of several *Taxus* species at varying basal levels of productivity (1–5 mg/L). We are therefore convinced that arachidonic acid is an effective elicitor. Since arachidonic acid specifically improved Taxol and not baccatin III yield, it apparently overcomes a limiting step in nitrogen supply to Taxol.

Concluding Remarks. We have examined the dynamics of taxane production and metabolic pathway compartmentation in *Taxus* sp. cell cultures by using enzyme, protein synthesis, and transport inhibitors. The results suggest that the source of IPP in taxane biosynthesis is plastidic. The differential influence of cycloheximide, streptomycin, ABT, and PCA on baccatin III and Taxol biosynthesis suggests that baccatin III may not be a direct precursor of Taxol. The results suggest a heretofore unsuspected branch point in the production of taxanes from which separate branches leading to the synthesis of baccatins and side chain added taxanes emerge. This information was built into conceptual models to describe carbon flow. Simple mathematical simulations of the suggested pathways revealed that baccatin III production could be limited by the activities of enzymes catalyzing the conversion of the branch point carbon precursor to baccatins. On the other hand, Taxol yield is alternately limited by the availability of nitrogen and carbon precursors and not by the side-chain transferase. The sensitivity of taxane production to PCA suggests that some of the oxidative chemistry in Taxol and baccatin III production could involve α-ketoglutarate dependent dioxygenases. Under current operating conditions (8 μM Taxol in 25 days), the yield of Taxol in the induction medium is limited by the availability of side chain precursors. This limitation probably is not at the level of primary nitrogen precursors such as phenylalanine, but possibly is due to the activities of key enzymes responsible for the synthesis of the side chain. The use of arachidonic acid as an elicitor successfully overcomes this limitation, presumably by stimulating this step(s).

We believe that the methods presented in this article could be generally useful in developing rational strategies for yield improvement. The nonintuitive nature of the model predictions suggest that this approach could be very useful especially when details of the biosynthetic pathway and subcellular compartmentation are not fully

Table 4. Elicitation of Taxane Production by Arachidonic Acid in PRO1-95 Cultures

treatment	baccatin III (μM)	taxol (μM)
control	5.60 ± 0.08	0.74 ± 0.22
arachidonic acid (5 μg/g)	7.50 ± 1.99	6.91 ± 0.64

[a] Elicitor was added on day 1, and cultures were harvested on day 15.

known. Certainly, the information and understanding gained in this study, combined with the details of Taxol biosynthesis that are continually becoming available, will facilitate the metabolic engineering of *Taxus* cell cultures for Taxol production.

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Note Added in Proof: A more complete discussion of effects of arachidonic acid and elicitors on taxane production appeared recently (Ciddi, V.; Srinivasan, V.; Shuler, M. L. Elicitation of *Taxus sp.* Cell Cultures for Production of Taxol. *Biotechnol. Lett.* **1995**, *17*, 1343–1346).

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